Structures of New Cyclic Diarylheptanoids and Inhibitors of Nitric Oxide Production from Japanese Folk Medicine *Acer nikoense*[1]

Toshio Morikawa, Jing Tao, Iwao Toguchida, Hisashi Matsuda, and Masayuki Yoshikawa*

Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8412, Japan

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Three new cyclic diarylheptanoids, acerosides B_1 and B_2 and aceroketoside, were isolated together with 20 known compounds from a Japanese folk medicine, the stem bark of *Acer nikoense*. The absolute stereostructures of the new compounds were determined on the basis of chemical and physicochemical evidence. In addition, the principal diarylheptanoid constituents were found to exhibit inhibitory activity on nitric oxide production in lipopolysaccharide-activated macrophages.

The stem bark of the Aceraceae plant *Acer nikoense* Maxim., which is indigenous to Japan, has been used as a folk medicine for hepatic disorders and eye diseases. As the chemical constituents of this plant, diarylheptanoids and phenolic compounds were characterized from the stem bark,[2] while tannin, coumarinolignans, flavonoids, sterols, and triterpenes were identified from the leaves and wood.[3] The methanolic extract of the stem bark was reported to show protective effects on hepatic injury induced by carbon tetrachloride in rats. Through bioassay-guided separation, (+)-rhododendrol was isolated as the active principal compound.[4]

As a part of our studies on bioactive constituents of natural medicines, we previously reported that the biphenyl-type diarylheptanoids from *Myrica rubra* Seib. et Zucc. showed inhibitory activities on the release of β-hexosaminidase from RBL-2H3 cells[5] and on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages,[6] as well as the absolute stereostructures of six new biphenyl-type diarylheptanoids. As a continuation of the characterization studies on bioactive diarylheptanoids, we have isolated three new cyclic diarylheptanoids named acerosides B_1 (**1**) and B_2 (**2**) and aceroketoside (**3**) from the stem bark of *A. nikoense*. This paper deals with the isolation and structure elucidation of three new cyclic diarylheptanoids (**1**−**3**). Furthermore, we describe the inhibitory effects of the principal diarylheptanoids on NO production in LPS-activated mouse peritoneal macrophages.

Results and Discussion

The methanolic extract from the stem bark of *A. nikoense* collected in Miyagi Prefecture, Japan, was partitioned into an ethyl acetate (EtOAc)−water mixture to furnish the EtOAc-soluble portion and aqueous phase. The aqueous phase was further extracted with *n*-butanol (*n*-BuOH) to give the *n*-BuOH-soluble and H_2O-soluble portions. The EtOAc-soluble and *n*-BuOH-soluble portions were subjected to normal-phase and reversed-phase silica gel column chromatography and repeated HPLC to give **1** (0.0003%, from the dried material), **2** (0.0003%), and **3** (0.0011%) together with acerogenins A[7,8] (**4**, 0.0078%), B[9] (**5**, 0.0035%), E[10] (**6**, 0.0005%), and K[11] (**7**, 0.0012%), (−)-centrolobol[8] (**8**, 0.0025%), (+)-rhododendrol[2] (0.019%), epirhododendrin[12] (0.0052%), 4-(4-hydroxyphenyl)-2-butanone[13] (0.0030%), (+)-catechin[14] (0.0032%), (−)-epicatechin[14] (0.0008%), clemiscosin D[15] (0.0009%), aquillochin[15] (0.0050%), scopoletin[16] (0.0070%), fraxetin[17] (0.0002%), fraxin[17] (0.0021%), vanillic acid[18] (0.0003%), 3,4,5-trimethoxybenzyl alcohol[18] (0.0011%), 2-hydroxy-5-methoxybenzaldehyde[18] (0.0002%), 4-hydroxy-2,6-dimethoxybenzoic acid[18] (0.0002%), and 2,4′-dihydroxy-5-carboxydiphenyl ether[19] (0.0006%).

Aceroside B_1 (**1**) was isolated as a white powder with negative optical rotation ($[\alpha]_D^{24}$ −94.0°). The positive-ion FABMS of **1** showed a quasimolecular ion peak at *m*/*z* 483 $[M + Na]^+$, while a quasimolecular ion peak was observed at *m*/*z* 459 $[M - H]^-$ and a fragment ion peak at *m*/*z* 297 $[M - C_6H_{11}O_5]^-$ in the negative-ion FABMS. The molecular formula $C_{25}H_{32}O_8$ of **1** was determined from the quasimolecular ion peak $[M + Na]^+$ and by HRFABMS measurement. The IR spectrum of **1** showed absorption bands at 3400, 1590, 1508, 1458, and 1074 cm^{-1} assignable to hydroxyl, aromatic ring, and ether functions, whereas in the UV spectrum, it showed an absorption maximum at 272 nm (log ϵ 3.24), which was suggestive of a diphenyl ether-type diarylheptanoid structure.[20] On acid hydrolysis with 1 M hydrochloric acid (HCl), **1** liberated D-glucose, which was identified by HPLC analysis using an optical rotation detector.[21] Enzymatic hydrolysis of **1** with β-glucosidase in acetate buffer (pH 4.4) yielded (−)-(*R*)-acerogenin B (**1a**), which was obtained as a colorless fine crystal with negative optical rotation ($[\alpha]_D^{24}$ −95.0°). The spectral data of **1a** were in agreement with those of racemic acerogenin B (**5**).[9] The absolute configuration at the 9-position in **1a** was determined by an application of the modified Mosher's method.[22] Namely, diazomethane methylation of **1a** furnished the 2-*O*-methyl derivative (**1b**), which was derivated to the 9-(*R*)- and 9-(*S*)-2-methoxy-2-trifluorophenylacetate (MTPA ester, **1c** and **1d**). As shown in Figure 1, the signals due to the protons attached to the 10, 11, 12, 13, 15, and 19-carbons in the 9-(*S*)-MTPA ester (**1d**) were observed at lower fields compared with those of the 9-(*R*)-MTPA ester (**1c**) [$\Delta\delta$: positive], while signals due to the protons of the 4, 6, 7, and 8-carbons in **1d** were observed at higher fields compared with those of **1c** [$\Delta\delta$: negative]. Consequently, the absolute stereostructure at the 9-position of **1a** has been determined to be the *R* configuration.

The ^1H NMR (pyridine-d_5) and ^{13}C NMR (Table 1) spectra[23] of **1** showed signals assignable to a (−)-(*R*)-acerogenin B moiety [δ 0.96, 1.48 (1H each, both m, H_2-10), 1.18, 1.48 (1H each, both m, H_2-11), 1.48, 1.68 (1H each, both m, H_2-12), 1.66, 1.73 (1H each, both m, H_2-8), 2.48, 2.64 (1H each, both m, H_2-13), 2.69, 2.99 (1H each, both m, H_2-7), 3.35 (1H, m, H-9), 5.95 (1H, d, J = 2.1 Hz, H-6), 6.74 (1H, dd, J = 2.1, 8.3 Hz, H-4), 6.91, 7.20 (1H each, both dd, J = 2.1, 8.3 Hz, H-16, 18), 7.11, 7.26 (1H each,

* To whom correspondence should be addressed. Tel: +81-75-595-4633. Fax: +81-75-595-4768. E-mail: shoyaku@mb.kyoto-phu.ac.jp.

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Chart 1

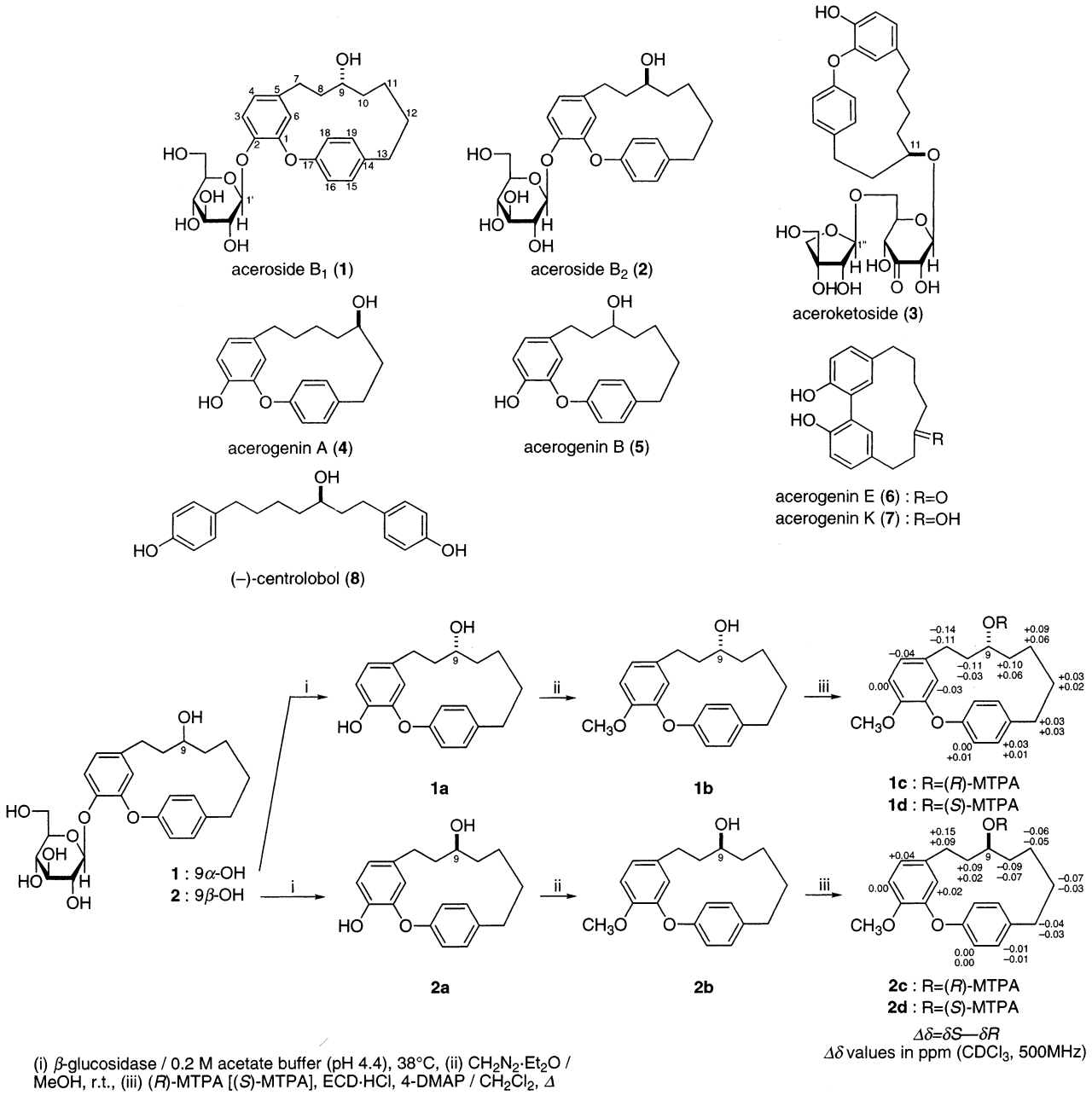

Figure 1.

both dd, J = 2.1, 8.3 Hz, H-15, 19), 7.54 (1H, d, J = 8.3 Hz, H-3)] and a β-D-glucopyranosyl part [δ 5.82 (1H, d, J = 7.3 Hz, H-1′)]. Comparison of the carbon signals in the ^{13}C NMR spectrum for **1** with those for **1a** disclosed a glycosilation shift around the 2-position of **1**. Furthermore, the position of the β-D-glucopyranosyl part in **1** was confirmed by the heteronuclear multiple bond connectivity (HMBC) experiment of **1**, which showed a long-range correlation between the 1′-proton and 2-carbon. On the basis of those findings, the structure of **1** including the absolute configuration was characterized to be as shown.

Aceroside B_2 (**2**) was also obtained as a white powder with positive optical rotation ($[\alpha]_D^{24}$ +24.5°). The molecular formula $C_{25}H_{32}O_8$, which was the same as that of **1**, was determined from the positive-ion and negative-ion FABMS and by HRFABMS analysis. That is, a quasimolecular ion peak was observed at m/z 483 $[M + Na]^+$ in the positive-ion FABMS, while the negative-ion FABMS showed a quasimolecular ion peak and a fragment ion peak at m/z 459 $[M - H]^-$ and 297 $[M - C_6H_{11}O_5]^-$. The IR spectrum of **2** showed absorption bands at 3400, 1590, 1508, 1458, and 1074 cm^{-1} assignable to hydroxyl, aromatic ring, and ether functions, whereas in the UV spectrum, it showed an absorption maximum at 273 nm (log ϵ 3.24), which was suggestive of a diphenyl ether-type diarylheptanoid structure.[20] The acid hydrolysis of **2** liberated D-glucose,[21] and the enzymatic hydrolysis of **2** furnished (+)-(*S*)-acerogenin B (**2a**) with positive optical rotation ($[\alpha]_D^{24}$ +84.1°), which was identified by comparison of the spectral data with those of racemic acerogenin B (**5**).[9] To confirm the absolute configuration at the 9-position, the modified Mosher's method was also applied to both of the 9-MTPA esters (**2c** and **2d**), which were prepared using the 2-methyl ether (**2b**) by the same procedure as that of **1**. As shown in Figure 1, the proton signals due to the 10, 11, 12, 13, 15, and 19-positions in the 9-(*S*)-MTPA ester (**2d**) appeared at a higher field than those of the 9-(*R*)-MTPA ester (**2c**) [$\Delta\delta$: negative], while the proton signals ascribable to the 4, 6, 7, and

Table 1. ^{13}C NMR Data for Acerosides B_1 (**1**) and B_2 (**2**), (−)-*R*-Acerogenin B (**1a**), (+)-*S*-Acerogenin B (**2a**), and Aceroketoside (**3**)

	1[a]	**2**[a]	**1a**[b]	**2a**[b]	**3**[a]
C-1	152.4	152.3	149.1	149.2	150.7
C-2	145.2	145.2	142.8	142.8	145.4
C-3	117.0	117.0	115.1	115.1	117.3
C-4	122.6	122.5	122.5	122.5	122.7
C-5	136.5	136.7	133.7	134.1	132.6
C-6	116.8	116.7	114.7	114.7	116.9
C-7	28.9	29.0	30.4	30.4	32.3
C-8	37.2	37.3	36.3	36.4	28.3
C-9	70.7	70.7	71.8	71.8	25.1
C-10	39.7	39.7	38.8	38.8	36.9
C-11	23.2	23.1	22.4	22.4	79.3
C-12	30.9	30.9	28.3	28.4	39.5
C-13	35.3	35.3	35.2	35.3	32.3
C-14	139.7	139.7	139.7	139.7	140.0
C-15	*132.2[c]	*132.1	*131.8	*131.8	*130.4
C-16	**123.8	**123.8	**123.5	**123.2	**123.5
C-17	156.4	156.4	155.3	155.3	156.8
C-18	**123.6	**123.6	**123.6	**123.3	**124.3
C-19	*131.1	*131.1	*130.7	*130.7	*132.5
C-1′	102.6	102.7			105.0
C-2′	74.9	75.0			78.4
C-3′	78.6	78.6			207.1
C-4′	71.4	71.3			74.0
C-5′	78.9	78.9			76.6
C-6′	62.5	62.5			68.5
C-1″					111.0
C-2″					77.6
C-3″					80.3
C-4″					75.1
C-5″					65.3

[a] Measured in pyridine-d_5 at 125 MHz. [b]Measured in $CDCl_3$ at 125 MHz. [c] *,** May be interchangeable within the same column.

8-positions of **2d** were observed at lower fields compared with those of **2c** [$\Delta\delta$: positive]. This evidence suggests the absolute stereostructure at the 9-position in **2a** to be the *S* configuration. The ^1H NMR (pyridine-d_5) and ^{13}C NMR (Table 1) spectra of **2** showed the presence of a (+)-(*S*)-acerogenin B moiety and a β-D-glucopyranosyl part [δ 5.85 (1H, d, J = 7.1 Hz, H-1′)]. Comparison of the ^{13}C NMR data for **2** with those for **2a** suggested a glycosidation shift at the 2-position, and the HMBC experiment on **2** showed a long-range correlation between the 1′-proton and 2-carbon. Consequently, the absolute stereostructure of aceroside B_2 (**2**) was determined to be the 9-isomer of **1**.

Aceroketoside (**3**), which was obtained as a white powder with negative optical rotation ($[\alpha]_D^{24}$ −82.2°), showed absorption bands at 3403, 1736, 1590, 1502, 1460, and 1041 cm^{-1} ascribable to hydroxyl, carbonyl, aromatic ring, and ether functions. The UV spectrum of **3** exhibited an absorption maximum at 278 nm (log ϵ 3.41). The positive-ion FABMS of **3** showed a quasimolecular ion peak at *m/z* 613 $[M + Na]^+$, while a quasimolecular ion peak was observed at *m/z* 589 $[M - H]^-$ together with a fragment ion peak at *m/z* 297 $[M - C_{11}H_{17}O_9]^-$ in the negative-ion FABMS, and HRFABMS analysis revealed the molecular formula of **3** to be $C_{30}H_{38}O_{12}$. Enzymatic hydrolysis of **3** with naringinase provided acerogenin A (**4**).[7,8] The ^1H NMR (pyridine-d_5) and ^{13}C NMR (Table 1) spectra[23] of **3** showed signals assignable to an acerogenin A moiety [δ 0.90, 1.08 (1H each, both m, H_2-9), 1.13, 1.50 (1H each, both m, H_2-10), 1.20, 1.38 (1H each, both m, H_2-8), 1.55, 2.34 (1H each, both m, H_2-12), 2.41, 2.49 (1H each, both m, H_2-7), 2.90, 3.08 (1H each, both m, H_2-13), 3.57 (1H, m, H-9), 5.94 (1H, d, J = 2.0 Hz, H-6), 6.71 (1H, dd, J = 2.0, 8.1 Hz, H-4), 7.22 (1H, d, J = 8.1 Hz, H-3), 7.23 (2H, dd, J = 2.0, 8.1 Hz, H-16, 18), 7.25, 7.69 (1H each, both dd, J = 2.0, 8.1 Hz, H-15, 19)], a 3-ketohexopyranosyl part [δ 4.06 (1H, m, H-5′), 4.27, 4.73 (1H each, both m, H_2-6′), 4.70 (1H, d, J = 7.5 Hz, H-2′), 4.76 (1H, d, J = 4.9 Hz, H-4′), 4.85 (1H, d, J = 7.8 Hz, H-1′)], and an apiofuranosyl part [δ 5.77 (1H, d, J = 2.4 Hz, H-1″)]. In the HMBC experiment on **3**, long-range correlations were observed between the following protons and carbons: H-11 and C-1′; H-1′ and C-11,3′; H-2′ and C-3′; H-4′ and C-3′; H-5′ and C-3′; H_2-6′ and C-1″; H-1″ and C-6′ (Figure 2). Comparison of the ^1H NMR and ^{13}C NMR data for **3** with those for aceroside III suggested that **3** was the 3′-keto derivative of aceroside III. Finally, reduction of **3** with sodium borohydride ($NaBH_4$) in methanol yielded a mixture of aceroside III and its 3′-epimer, which was subjected to acid hydrolysis with 1 M HCl to give D-glucose, D-allose, and D-apiose. On the basis of this evidence, the structure of aceroketoside (**3**) was elucidated as shown.

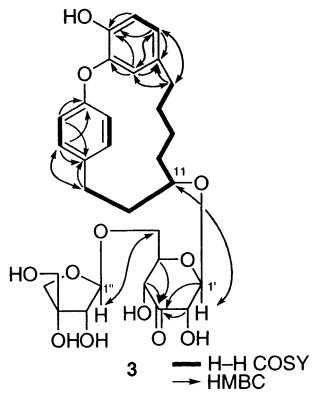

Figure 2.

The inorganic free radical NO has been implicated in physiological and pathological processes such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation.[24] NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). In the NOS family, inducible NOS in particular is involved in pathological overproduction of NO and can be expressed in response to pro-inflammatory agents such as interleukin-1β, tumor necrosis factor-α, and LPS in various cell types including macrophages, endothelial cells, and smooth muscle cells.

As part of our studies to characterize the bioactive components of natural medicines, we have reported several NO production inhibitors, i.e., higher unsaturated fatty acids,[25] polyacetylenes,[26,27] coumarins,[26] flavonoids,[27] stilbenes,[28] lignans,[29] sesquiterpenes,[30−32] diterpenes,[33] triterpenes,[6,34] and biphenyl-type diarylheptanoids.[6] As a continuatiion of these studies, we examined the effects of the five constituents from the stem bark of *A. nikoense* on NO production from LPS-activated macrophages, and the results are summarized in Table 2.

Five diarylheptanoid constituents were examined and found to show inhibitory activity (IC_{50} = 24−88 μM; Table 2) without cytotoxic effects in the MTT assay (data not shown). Among them, the inhibitory activities of biphenyl-type diarylheptanoids, acerogenins E (**6**, IC_{50} = 24 μM) and K (**7**, IC_{50} = 25 μM), were equivalent to that of N^G-monomethyl-L-arginine (L-NMMA), a nonselective NOS inhibitor (IC_{50} = 28 μM). Comparison of the inhibitory activity for acerogenins A (**4**, IC_{50} = 74 μM) and B (**5**, IC_{50} = 88 μM) and (−)-centrolobol (**8**, IC_{50} = 73 μM) with those of **6** and **7** suggested that biphenyl-type diarylheptanoids had stronger NO production inhibitory activity than diphenyl ether-type and straight chain-type diarylheptanoids.

Experimental Section

General Experimental Procedures. The following instruments were used to obtain physical data: specific rota-

Table 2. Inhibitory Effects of Constituents from *A. nikoense* on NO Production in LPS-Activated Mouse Peritoneal Macrophages[a]

	inhibition (%)						
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	IC_{50} (μM)
acerogenin A (**4**)	0.0 ± 0.9	6.7 ± 0.7	5.8 ± 2.6	11.6 ± 2.1**[,b]	22.2 ± 1.9**	61.4 ± 0.9**	74
acerogenin B (**5**)	0.0 ± 1.4	1.9 ± 1.1	−0.5 ± 3.3	−0.2 ± 2.1	17.6 ± 1.3**	54.1 ± 2.1**	88
acerogenin E (**6**)	0.0 ± 2.1	0.1 ± 3.8	3.3 ± 2.5	13.6 ± 3.1**	59.4 ± 1.2**	81.2 ± 1.8**	24
acerogenin K (**7**)	0.0 ± 3.8	6.6 ± 3.6	2.1 ± 4.4	24.3 ± 3.7**	56.6 ± 2.9**	91.3 ± 0.9**	25
(−)-centrolobol (**8**)	0.0 ± 1.9	−1.3 ± 2.6	1.3 ± 3.0	−1.1 ± 2.3	10.7 ± 1.4**	67.2 ± 0.7**	73
L-NMMA	0.0 ± 1.1	4.4 ± 2.0	2.0 ± 1.6	17.7 ± 2.8**	52.3 ± 1.5**	79.2 ± 0.9**	28

[a] Each value represents the mean ± SEM ($N = 4$). [b] Significantly different from the control, $^{**}p < 0.01$.

tions, Horiba SEPA-300 digital polarimeter ($l = 5$ cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; EIMS and HREIMS, JEOL JMS-GCMATE mass spectrometer; FABMS and HRFABMS, JEOL JMS-SX 102A mass spectrometer; ^{1}H NMR spectra, JEOL LNM-500 (500 MHz) spectrometer; ^{13}C NMR spectra, JEOL LNM-500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; HPLC, Shimadzu RID-6A refractive index detector.

The following experimental conditions were used for chromatography: normal-phase silica gel column chromatography, silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150−350 mesh); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100−200 mesh); TLC, precoated TLC plates with silica gel $60F_{254}$ (Merck, 0.25 mm) (normal-phase) and silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed-phase); reversed-phase HPTLC, precoated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm); detection was achieved by spraying with 1% $Ce(SO_4)_2$−10% aqueous H_2SO_4 followed by heating.

Plant Material. The stem bark of *Acer nikoense* was collected in Miyagi Prefecture, Japan, and identified by one of the authors (M.Y.). A voucher of the plant is on file in our laboratory.

Extraction and Isolation. The dried stem bark of *A. nikoense* (8.5 kg) was finely cut and extracted three times with methanol (MeOH) under reflux for 3 h. Evaporation of the solvent under reduced pressure provided the methanolic extract (232 g, 2.7%). The methanolic extract (196 g) was partitioned in an EtOAc−H_2O (1:1, v/v) mixture. The aqueous layer was extracted with *n*-BuOH, and removal of the solvent in vacuo from the EtOAc-, *n*-BuOH-, and H_2O-soluble portions yielded 60 g (0.8%), 103 g (1.4%), and 34 g (0.5%) of the residue, respectively.

Normal-phase silica gel column chromatography [BW-200 (Fuji Silysia Co., Ltd., 1.6 kg), $CHCl_3$−MeOH (20:1−10:1, v/v)−$CHCl_3$−MeOH−H_2O (7:3:1, lower layer-6:4:1, v/v/v)−MeOH] of the *n*-BuOH-soluble portion (80 g) gave six fractions [Fr. 1 (2.6 g), 2 (13.3 g), 3 (17.0 g), 4 (30.4 g), 5 (10.2 g), 6 (6.5 g)]. Fraction 2 (13.3 g) was separated by reversed-phase silica gel column chromatography [Chromatorex ODS DM1020T (Fuji Silysia Co., Ltd., 300 g), MeOH−H_2O (50:50−60:40−70:30, v/v)−MeOH] to furnish seven fractions [Fr. 2-1 (0.20 g), 2-2 (0.57 g), 2-3 (2.80 g), 2-4 (1.00 g), 2-5 (0.70 g), 2-6 (0.50 g), 2-7 (7.53 g)]. Fraction 2-1 (0.20 g) was further separated by HPLC [YMC-Pack ODS-A (YMC Co., Ltd., Kyoto, Japan, 250 × 20 mm i.d.), MeOH−H_2O (30:70, v/v)] to give vanillic acid (4 mg, 0.0003%). Fraction 2-2 (0.57 g) was separated by HPLC [MeOH−H_2O (45:55, v/v)] to give (+)-rhododendrol (57 mg, 0.0099%), 4-(4-hydroxyphenyl)-2-butanone (12 mg, 0.0022%), and scopoletin (20 mg, 0.0035%). Fraction 2-3 (2.80 g) was separated by HPLC [MeOH−H_2O (35:65, v/v)] to give cleomoiscosin D (28 mg, 0.0009%) and aquillochin (292 mg, 0.0050%). Fraction 2-5 (0.61 g) was purified by HPLC [MeOH−H_2O (35:65, v/v)] to give acerogenins A (**4**, 122 mg, 0.0022%) and B (**5**, 58 mg, 0.0010%) and (−)-centrolobol (**8**, 34 mg, 0.0006%). Fraction 2-6 (0.40 g) was subjected to HPLC [MeOH−H_2O (35:65, v/v)] to furnish acerogenins E (**6**, 10 mg, 0.0002%) and K (**7**, 26 mg, 0.0004%). Fraction 3 (15.0 g) was subjected to reversed-phase silica gel column chromatography [450 g, MeOH−H_2O (30:70−50:50−70:30, v/v)−MeOH] to furnish eight fractions [Fr. 3-1 (1.68 g), 3-2 (0.38 g), 3-3 (0.72 g), 3-4 (1.06 g), 3-5 (3.06 g), 3-6 (1.95 g), 3-7 (0.87 g), 3-8 (5.28 g)]. Fraction 3-3 (0.72 g) was further purified by HPLC [MeOH−H_2O (30:70, v/v)] to give epirhododendrin (265 mg, 0.0052%), (+)-catechin (163 mg, 0.0032%), (−)-epicatechin (42 mg, 0.0008%), and fraxin (109 mg, 0.0021%). Fraction 3-6 (1.95 g) was purified by HPLC [(1) MeOH−H_2O (60:40, v/v), (2) CH_3CN−H_2O (25:75, v/v)] to give acerosides B_1 (**1**, 11 mg, 0.0003%) and B_2 (**2**, 12 mg, 0.0003%) and aceroketoside (**3**, 61 mg, 0.0011%).

The EtOAc-soluble fraction (40 g) was subjected to normal-phase silica gel column chromatography [1.2 kg, *n*-hexane−EtOAc (1:1)−MeOH] to give three fractions [Fr. 1 (2.7 g), Fr. 2 (3.1 g), Fr. 3 (34.2 g)]. Fraction 2 (3.1 g) was separated by reversed-phase silica gel column chromatography [90 g, MeOH−H_2O (30:70−70:30, v/v)−MeOH] to give five fractions [Fr. 2-1 (0.47 g), 2-2 (0.28 g), 2-3 (0.76 g), 2-4 (0.67 g), 2-5 (0.92 g)]. Fraction 2-2 (0.28 g) was further purified by HPLC [MeOH−H_2O (40:60, v/v)] to give fraxetin (9 mg, 0.0002%), 3,4,5-trimethoxybenzyl alcohol (55 mg, 0.0011%), 2-hydroxy-5-methoxybenzaldehyde (9 mg, 0.0002%), and 4-hydroxy-2,6-dimethoxybenzoic acid (11 mg, 0.0002%). Fraction 2-3 (0.76 g) was subjected to HPLC [MeOH−H_2O (40:60 or 45:55, v/v)] to give (+)-rhododendrol (424 mg, 0.0087%), 4-(4-hydroxyphenyl)-2-butanone (40 mg, 0.0008%), scopoletin (167 mg, 0.0035%), and 2,4′-dihydroxy-5-carboxydiphenyl ether (28 mg, 0.0006%). Fraction 2-4 (0.67 g) was purified by HPLC [MeOH−H_2O (65:35, v/v)] to furnish **4** (248 mg, 0.0056%), **5** (113 mg, 0.0025%), **6** (13 mg, 0.0003%), **7** (35 mg, 0.0008%), and **8** (84 mg, 0.0019%).

The known compounds were identified by comparison of their physical data ($[\alpha]_D$, IR, ^{1}H NMR, ^{13}C NMR, MS) with reported values,[2,7−13,15−17,19] authentic samples,[14] or commercial samples.[18]

Aceroside B_1 (1): a white powder; $[\alpha]_D^{24}$ −94.0° (*c* 0.40, EtOH); UV (EtOH) λ_{max} (log ϵ) 272 (3.24); IR (KBr) ν_{max} 3400, 2928, 1590, 1508, 1458, 1074 cm^{-1}; ^{1}H NMR (pyridine-d_5, 500 MHz) δ 0.96, 1.48 (1H each, both m, H_2-10), 1.18, 1.48 (1H each, both m, H_2-11), 1.48, 1.68 (1H each, both m, H_2-12), 1.66, 1.73 (1H each, both m, H_2-8), 2.48, 2.64 (1H each, both m, H_2-13), 2.69, 2.99 (1H each, both m, H_2-7), 3.35 (1H, m, H-9), 5.82 (1H, d, $J = 7.3$ Hz, H-1′), 5.95 (1H, d, $J = 2.1$ Hz, H-6), 6.74 (1H, dd, $J = 2.1$, 8.3 Hz, H-4), 6.91, 7.20 (1H each, both dd, $J = 2.1$, 8.3 Hz, H-16, 18), 7.11, 7.26 (1H each, both dd, $J = 2.1$, 8.3 Hz, H-15, 19), 7.54 (1H, d, $J = 8.3$ Hz, H-3); ^{13}C NMR data, see Table 1; positive-ion FABMS *m/z* 483 $[M + Na]^+$; negative-ion FABMS *m/z* 459 $[M - H]^-$, 297 $[M - C_6H_{11}O_5]^-$; HR-FABMS *m/z* 483.1986 (calcd for $C_{25}H_{32}O_8Na$ $[M + Na]^+$, 483.1995).

Aceroside B_2 (2): white powder; $[\alpha]_D^{24}$ +24.5° (*c* 0.50, EtOH); UV (EtOH) λ_{max} (log ϵ) 273 (3.24); IR (KBr) ν_{max} 3400, 2926, 1590, 1508, 1458, 1074 cm^{-1}; ^{1}H NMR (pyridine-d_5, 500 MHz) δ 0.98, 1.47 (1H each, both m, H_2-10), 1.18, 1.45 (1H each, both m, H_2-11), 1.51, 1.65 (1H each, both m, H_2-12), 1.67, 1.75 (1H each, both m, H_2-8), 2.50, 2.63 (1H each, both m, H_2-13), 2.68, 3.00 (1H each, both m, H_2-7), 3.37 (1H, m, H-9), 5.85 (1H, d, $J = 7.1$ Hz, H-1′), 5.95 (1H, d, $J = 2.1$ Hz, H-6), 6.73 (1H, dd, $J = 2.1$, 8.3 Hz, H-4), 6.91, 7.21 (1H each, both dd, $J = 2.1$, 8.3 Hz, H-16, 18), 7.11, 7.26 (1H each, both dd, $J = 2.1$, 8.3 Hz, H-15, 19), 7.54 (1H, d, $J = 8.3$ Hz, H-3); ^{13}C NMR data, see Table 1; positive-ion FABMS *m/z* 483 $[M + Na]^+$; negative-ion FABMS *m/z* 459 $[M - H]^-$, 297 $[M - C_6H_{11}O_5]^-$; HR-FABMS *m/z* 483.2002 (calcd for $C_{25}H_{32}O_8Na$ $[M + Na]^+$, 483.1995).

Aceroketoside (3): white powder; $[\alpha]_D^{24}$ −82.2° (*c* 0.20, EtOH); UV (EtOH) λ_{max} (log ϵ) 278 (3.41); IR (KBr) ν_{max} 3403, 2932, 1736, 1590, 1502, 1460, 1041 cm^{-1}; ^{1}H NMR (pyridine-d_5, 500 MHz) δ 0.90, 1.08 (1H each, both m, H_2-9), 1.13, 1.50 (1H each, both m, H_2-10), 1.20, 1.38 (1H each, both m, H_2-8), 1.55, 2.34 (1H each, both m, H_2-12), 2.41, 2.49 (1H each, both m, H_2-7), 2.90, 3.08 (1H each, both m, H_2-13), 3.57 (1H, m, H-9), 4.06 (1H, m, H-5′), 4.27, 4.73 (1H each, both m, H_2-6′), 4.70 (1H, d, J = 7.5 Hz, H-2′), 4.76 (1H, d, J = 4.9 Hz, H-4′), 4.85 (1H, d, J = 7.8 Hz, H-1′), 5.77 (1H, d, J = 2.4 Hz, H-1″), 5.94 (1H, d, J = 2.0 Hz, H-6), 6.71 (1H, dd, J = 2.0, 8.1 Hz, H-4), 7.22 (1H, d, J = 8.1 Hz, H-3), 7.23 (2H, dd, J = 2.0, 8.1 Hz, H-16, 18), 7.25, 7.69 (1H each, both dd, J = 2.0, 8.1 Hz, H-15, 19); ^{13}C NMR data, see Table 1; positive-ion FABMS *m*/*z* 613 $[M + Na]^+$; negative-ion FABMS *m*/*z* 589 $[M - H]^-$, 297 $[M - C_{11}H_{17}O_9]^-$; HRFABMS *m*/*z* 613.2269 (calcd for $C_{30}H_{38}O_{12}$-Na $[M + Na]^+$, 613.2261).

Acid Hydrolysis of Acerosides B_1 (1) and B_2 (2). A solution of **1** or **2** (5 mg each) in 1 M HCl (0.5 mL) was heated under reflux for 2 h. After cooling, the reaction mixture was poured into ice−water and neutralized with Amberlite IRA-400 (OH^- form), and the resin was removed by filtration. Then, the filtrate was extracted with EtOAc. The aqueous layer was subjected to HPLC analysis under the following conditions: HPLC column, Shodex Asahipak NH-2P-50-4E, 4.6 mm i.d. × 250 mm (Showa Denko Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan)]; mobile phase, CH_3CN-H_2O (75:25, v/v); flow rate 0.8 mL/min; column temperature, room temperature. Identification of D-glucose present in the aqueous layer was carried out by comparison of its retention time and optical rotation with that of an authentic sample. t_R: 11.1 min (D-glucose, positive optical rotation).

Enzymatic Hydrolysis of Acerosides B_1 (1) and B_2 (2). A solution of **1** (6.0 mg) or **2** (7.0 mg) in 0.2 M acetate buffer (pH 4.4, 2.0 mL) was treated with β-glucosidase (10 mg from almond, Oriental Yeast Co., Ltd., Tokyo, Japan), and the mixture was stirred at 38 °C for 4 h. After the addition of EtOH to the reaction mixture, the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography [0.5 g, *n*-hexane−EtOAc (1:1, v/v)] to give (−)-(*R*)-acerogenin B (**1a**, 3.5 mg, 90%) or (+)-(*S*)-acerogenin B (**2a**, 3.9 mg, 86%).

(−)-(*R*)-Acerogenin B (1a): colorless fine crystals; mp 182.0−184.5 °C (from MeOH); $[\alpha]_D^{22}$ −95.0° (*c* 0.10, EtOH); UV (EtOH) λ_{max} (log ϵ) 276 (3.32); IR (KBr) ν_{max} 3460, 2951, 1592, 1507, 1456 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.79, 1.23 (1H each, both m, H_2-10), 1.00, 1.30 (1H each, both m, H_2-11), 1.50 (2H, m, H_2-8), 1.53, 1.76 (1H each, both m, H_2-12), 2.57, 2.63 (1H each, both m, H_2-7), 2.63, 2.80 (1H each, both m, H_2-13), 3.07 (1H, m, H-9), 5.57 (1H, d, J = 1.8 Hz, H-6), 6.63 (1H, dd, J = 1.8, 8.2 Hz, H-4), 6.84 (1H, d, J = 8.2 Hz, H-3), 6.93, 7.13 (1H each, both dd, J = 2.5, 8.2 Hz, H-16, 18), 7.23, 7.30 (1H each, both dd, J = 2.1, 8.2 Hz, H-15, 19); ^{13}C NMR data, see Table 1; EIMS *m*/*z* 298 $[M^+]$ (100), 280 $[M^+ - H_2O]$ (75); HREIMS: *m*/*z* 298.1561 (calcd for $C_{19}H_{22}O_3$, 298.1569).

(+)-(*S*)-Acerogenin B (2a): colorless fine crystals; mp 181.3−183.4 °C (from MeOH); $[\alpha]_D^{22}$ +84.1° (*c* 0.20, EtOH); UV (EtOH) λ_{max} (log ϵ) 276 (3.34); IR (KBr) ν_{max} 3400, 2951, 1590, 1508, 1456 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.80, 1.22 (1H each, both m, H_2-10), 1.00, 1.30 (1H each, both m, H_2-11), 1.48 (2H, m, H_2-8), 1.50, 1.76 (1H each, both m, H_2-12), 2.57, 2.62 (1H each, both m, H_2-7), 2.63, 2.80 (1H each, both m, H_2-13), 3.07 (1H, m, H-9), 5.57 (1H, d, J = 2.0 Hz, H-6), 6.63 (1H, dd, J = 2.0, 8.2 Hz, H-4), 6.84 (1H, d, J = 8.2 Hz, H-3), 6.93, 7.14 (1H each, both dd, J = 2.5, 8.3 Hz, H-16, 18), 7.25, 7.30 (1H each, both dd, J = 2.2, 8.3 Hz, H-15, 19); ^{13}C NMR data, see Table 1; EIMS *m*/*z* 298 $[M^+]$ (100), 280 $[M^+ - H_2O]$ (70); HREIMS *m*/*z* 298.1564 (calcd for $C_{19}H_{22}O_3$, 298.1569).

Diazomethane Methylation of 1a and 2a. A solution of **1a** (3.0 mg) or **2a** (3.5 mg) in MeOH (2.0 mL) was treated with ethereal diazomethane (CH_2N_2·Et_2O) until the yellow color persisted. The reaction solution was stirred at room temperature for 2 h. Removal of the solvent under reduced pressure furnished a residue, which was purified by silica gel column chromatography [0.5 g, *n*-hexane−EtOAc (5:1, v/v)] to give **1b** (3.0 mg, 96%) or **2b** (3.7 mg, quant.).

1b: a white powder; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.85, 1.22 (1H each, both m, H_2-10), 1.00, 1.28 (1H each, both m, H_2-11), 1.49 (2H, m, H_2-8), 1.55, 1.74 (1H each, both m, H_2-12), 2.58, 2.64 (1H each, both m, H_2-7), 2.64, 2.78 (1H each, both m, H_2-13), 3.13 (1H, m, H-9), 3.95 (3H, s, OCH_3), 5.57 (1H, d, J = 2.2 Hz, H-6), 6.67 (1H, dd, J = 2.2, 8.4 Hz, H-4), 6.82 (1H, d, J = 8.4 Hz, H-3), 6.97, 7.13 (1H each, both dd, J = 2.4, 8.2 Hz, H-16, 18), 7.22, 7.28 (1H each, both dd, J = 2.1, 8.2 Hz, H-15, 19); EIMS *m*/*z* 312 $[M^+]$ (68), 294 $[M^+ - H_2O]$ (100); HREIMS *m*/*z* 312.1708 (calcd for $C_{20}H_{24}O_3$, 312.1725).

2b: a white powder; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.85, 1.22 (1H each, both m, H_2-10), 1.05, 1.28 (1H each, both m, H_2-11), 1.49 (2H, m, H_2-8), 1.56, 1.74 (1H each, both m, H_2-12), 2.58, 2.64 (1H each, both m, H_2-7), 2.64, 2.80 (1H each, both m, H_2-13), 3.13 (1H, m, H-9), 3.95 (3H, s, OCH_3), 5.57 (1H, d, J = 2.2 Hz, H-6), 6.67 (1H, dd, J = 2.2, 8.1 Hz, H-4), 6.82 (1H, d, J = 8.1 Hz, H-3), 6.97, 7.13 (1H each, both dd, J = 2.5, 8.1 Hz, H-16, 18), 7.23, 7.28 (1H each, both dd, J = 2.2, 8.1 Hz, H-15, 19); EIMS *m*/*z* 312 $[M^+]$ (100), 294 $[M^+ - H_2O]$ (39); HREIMS *m*/*z* 312.1717 (calcd for $C_{20}H_{24}O_3$, 312.1725).

Preparation of the (*R*)-MTPA Esters (1c, 2c) and (*S*)-MTPA Esters (1d, 2d) from 1b and 2b. A solution of **1b** (1.0 mg) or **2b** (1.0 mg) in CH_2Cl_2 (1.0 mL) was treated with (*R*)-2-methoxy-2-trifluoromethylphenylacetic acid [(*R*)-MTPA, 7.5 mg] in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC·HCl, 6.2 mg) and 4-(dimethylamino)-pyridine (4-DMAP, 2.0 mg), and the mixture was stirred under reflux for 6 h. After cooling, the reaction mixture was poured into ice−water, and the whole was extracted with AcOEt. The AcOEt extract was successively washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ powder and filtrated. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was purified by silica gel column chromatography [0.5 g, *n*-hexane−EtOAc (5:1, v/v)] to give **1c** (1.4 mg, 83%) or **2c** (1.3 mg, 77%), respectively. Using a similar procedure, (*S*)-MTPA esters [**1d** (1.5 mg, 89%) or **2d** (1.0 mg, 59%)] were obtained from **1b** (1.0 mg) and **2b** (1.0 mg) using (*S*)-MTPA (7.5 mg), EDC·HCl (6.2 mg), and 4-DMAP (2.0 mg).

1c: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.93, 1.40 (1H each, both m, H_2-10), 1.03, 1.39 (1H each, both m, H_2-11), 1.63, 1.73 (1H each, both m, H_2-8), 1.45, 1.72 (1H each, both m, H_2-12), 2.48, 2.51 (1H each, both m, H_2-7), 2.63, 2.73 (1H each, both m, H_2-13), 3.50, 3.96 (3H each, both s, OCH_3), 4.64 (1H, m, H-9), 5.52 (1H, d, J = 2.1 Hz, H-6), 6.62 (1H, dd, J = 2.1, 8.3 Hz, H-4), 6.80 (1H, d, J = 8.3 Hz, H-3), 7.05, 7.13 (1H each, both dd, J = 2.5, 8.3 Hz, H-16, 18), 7.23, 7.29 (1H each, both dd, J = 2.2, 8.3 Hz, H-15, 19), 7.39−7.49 (5H, m, Ph-H).

1d: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.03, 1.46 (1H each, both m, H_2-10), 1.12, 1.45 (1H each, both m, H_2-11), 1.60, 1.62 (1H each, both m, H_2-8), 1.48, 1.74 (1H each, both m, H_2-12), 2.34, 2.40 (1H each, both m, H_2-7), 2.66, 2.76 (1H each, both m, H_2-13), 3.50, 3.96 (3H each, both s, OCH_3), 4.64 (1H, m, H-9), 5.49 (1H, d, J = 2.1 Hz, H-6), 6.58 (1H, dd, J = 2.1, 8.3 Hz, H-4), 6.80 (1H, d, J = 8.3 Hz, H-3), 7.06, 7.13 (1H each, both dd, J = 2.5, 8.3 Hz, H-16, 18), 7.26, 7.30 (1H each, both dd, J = 2.2, 8.3 Hz, H-15, 19), 7.39−7.49 (5H, m, Ph-H).

2c: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.02, 1.45 (1H each, both m, H_2-10), 1.08, 1.43 (1H each, both m, H_2-11), 1.60, 1.63(1H each, both m, H_2-8), 1.52, 1.75 (1H each, both m, H_2-12), 2.33, 2.42 (1H each, both m, H_2-7), 2.66, 2.76 (1H each, both m, H_2-13), 3.50, 3.96 (3H each, both s, OCH_3), 4.64 (1H, m, H-9), 5.49 (1H, d, J = 2.1 Hz, H-6), 6.58 (1H, dd, J = 2.1, 8.3 Hz, H-4), 6.81 (1H, d, J = 8.3 Hz, H-3), 7.06, 7.13 (1H each, both dd, J = 2.3, 8.3 Hz, H-16, 18), 7.24, 7.30 (1H each, both dd, J = 2.3, 8.3 Hz, H-15, 19), 7.39−7.49 (5H, m, Ph-H).

2d: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.93, 1.38 (1H each, both m, H_2-10), 1.03, 1.37 (1H each, both m, H_2-11), 1.45, 1.72 (1H each, both m, H_2-12), 1.62, 1.72 (1H each, both m, H_2-8), 2.48, 2.51 (1H each, both m, H_2-7), 2.63, 2.72 (1H each, both m, H_2-13), 3.50, 3.96 (3H each, both s, OCH_3), 4.64 (1H, m, H-9), 5.51 (1H, d, J = 2.1 Hz, H-6), 6.62 (1H, dd, J = 2.1, 8.3 Hz, H-4), 6.81 (1H, d, J = 8.3 Hz, H-3), 7.06, 7.13 (1H each, both dd, J

= 2.3, 8.3 Hz, H-16, 18), 7.23, 7.29 (1H each, both dd, J = 2.1, 8.3 Hz, H-15, 19), 7.39−7.49 (5H, m, Ph-H).

$NaBH_4$ Reduction and Acid Hydrolysis of Aceroketoside (3). A solution of **3** (5.0 mg) in MeOH (1.0 mL) was treated with $NaBH_4$ (1.0 mg), and the mixture was stirred at room temperature for 1 h. The reaction mixture was quenched with acetone, and then removal of the solvent under reduced pressure yielded a reduction mixture. Aceroside III[35] was detected in the reduction mixture by TLC analysis [solvent: $CHCl_3$–MeOH−H_2O (10:3:1, lower layer, v/v/v)]. Then, a solution of the reduction mixture in 1 M HCl (0.5 mL) was heated under reflux for 2 h. After cooling, the reaction mixture was poured into ice−water and neutralized with Amberlite IRA-400 (OH^- form), and the residue was removed by filtration. Then the reaction mixture was extracted with EtOAc. The aqueous layer of the above mixture was subjected to HPLC analysis under the following conditions: HPLC column, Shodex Asahipak NH-2P-50−4E, 4.6 mm i.d. × 250 mm; detection, optical rotation; mobile phase, CH_3CN-H_2O (75:25, v/v); flow rate 0.8 mL/min; column temperature, room temperature. Identification of D-apiose,[36] D-allose, and D-glucose present in the aqueous layer was carried out by comparison of their retention time and optical rotation with those of authentic samples. t_R: 6.4 min (D-apiose, positive optical rotation), 9.7 min (D-allose, positive optical rotation), and 11.1 min (D-glucose, positive optical rotation).

Enzymatic Hydrolysis of Aceroketoside (3). A solution of **3** (8.0 mg) in 0.2 M acetate buffer (pH 4.8, 2.0 mL) was treated with naringinase (10 mg from *Penicillium decumbens*, Sigma Co., Ltd., St. Louis, MO), and the mixture was stirred at 38 °C for 4 day. After the addition of EtOH to the reaction mixture, the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography [0.5 g, $CHCl_3-MeOH-H_2O$ (20:3:1, lower layer, v/v/v)] to give acerogenin A (**4**, 1.7 mg, 42%).[10]

NO Production from Macrophages Stimulated by LPS. Peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice by washing with 6−7 mL of ice-cold phosphate-buffered saline (PBS), and cells (5×10^5 cells/well) were suspended in 200 μL of RPMI 1640 supplemented with 5% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) and pre-cultured in 96-well microplates at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing the cells with PBS, and the adherent cells (more than 95% macrophages as determined by Giemsa staining) were cultured in fresh medium containing 10 μg/mL LPS and test compound (10, 30, and 100 μM) for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent.

Cytotoxicity was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric assay. Briefly, after 20 h incubation with test compounds, MTT (10 μL, 5 mg/mL in PBS) solution was added to the wells. After 4 h culture, the medium was removed, and 2-propanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm (reference, 655 nm). N^G-Monomethyl-L-arginine (L-NMMA) was used as a reference compound. Each test compound was dissolved in dimethyl sulfoxide (DMSO), and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated by the following formula, and the IC_{50} was determined graphically (N = 4):

$$\text{inhibition (\%)} = \frac{A - B}{A - C} \times 100$$

$A - C$: NO_2^- concentration (μM) [A: LPS (+), sample (−); B: LPS (+), sample (+); C: LPS (−), sample (−)].

Statistics. Values are expressed as means ± SEM. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

References and Notes

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